Platform F: Protein-Nucleic Acid Interactions I

62-Plat
Nucleic Acid Translocation By Hepatitis C Virus Helicase NS3h Is Dependent on Sugar and Base Moieties
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The NS3 helicase (NS3h) of hepatitis C virus (HCV) is a 3′ to 5′ SF2 RNA and DNA helicase that is essential for the replication of HCV. We have examined the kinetic mechanism of translocation of NS3h along single stranded nucleic acid with bases rU, dU and dT and found that the rate of translocation is dependent upon both base and sugar moieties. We find that the approximate rates of translocation are 3 nt/s (oligo-dT), 35 nt/s (oligo-dU), and 42 nt/s (oligo-rU). These macroscopic translocation rates correspond well to differences in the binding affinity of the translocating NS3h protein to the respective substrates. The values of K_M for NS3h translocating at a saturating ATP concentration are: 3.3 ($\pm$ 0.4) μM nucleotide (poly-dT), 27 ($\pm$ 2) μM nucleotide (poly-dU), and 36 ($\pm$ 2) μM nucleotide (poly-rU). Despite the differences in translocation rates and binding affinities, the ATP coupling stoichiometry for NS3h translocation is identical for all three substrates, with a value of ~2 nt per ATP consumed. The identical periodicity of ATP consumption implies a similar mechanism for NS3h translocation along each substrate. This data, together with our independently determined values of K_D for NS3h binding to poly-dT (220 $\pm$ 20 nM nucleotide) and poly-dU (430 $\pm$ 30 nM nucleotide), suggest that the differences in the macroscopic translocation rates may be explained by differences in the entropic contribution to the binding free energy of NS3h to the different nucleic acid substrates. This conclusion is consistent with observations from a previously published crystal structure of NS3h in complex with a short oligonucleotide (Kim, et al (1998) Structure 6:89-100).

63-Plat
Protein-mRNA Interactions Observed in Living Cells By Dual-Color Fluorescence Fluctuation Spectroscopy
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RNA binding protein controls the localization, translation and degradation of mRNA of specific proteins, therefore exerting a key influence on the cell metabolism, motility and differentiation. The zipcode binding protein 1 (ZBP1) binds to the 3′ untranslated region (UTR) of beta-actin mRNA and regulates its translation. Quantitative characterization of the interaction between mRNA and protein is crucial to dissect this regulation mechanism. We have developed a dual-color fluorescence fluctuation spectroscopy (FFS) technique to study the interaction between mRNA and protein directly in living cells. FFS determines the brightness, concentration and diffusion time of fluorescent particles from the intensity bursts generated by individual particles passing through a small observation volume. Dual-color FFS distinguishes fluorescent species by brightness and diffusion time as well as the fluorescence color. In this study, we apply dual-color FFS to study the interaction between beta-actin mRNA and ZBP1. The endogenous mouse beta-actin mRNA is visualized by incorporating 24 MS2 stem-loops in the 3′ UTR, which are specifically bound by fluorescently labeled MS2 coat protein (MCP). We have developed a tandem dimeric MCP system that is particularly suitable for FFS brightness analysis. Since multiple MCPs bind to a single mRNA, the brightness of mRNA is significantly higher than that of free MCP, which allows us to readily resolve mRNA from free MCPs. Furthermore, ZBP1 is labeled with a different color and dual-color FFS directly extracts the interacting mRNA-ZBP1 species. The interaction is visualized by brightness signatures and the binding affinity is extracted by measuring different cells with varying concentrations of ZBP1. This work is supported by National Institute of Health EB2060.

64-Plat
Hybridization Kinetics Is Different Inside Cells
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It is generally expected that the kinetics of reactions inside living cells differs from the situation in bulk solutions. Macromolecular crowding as well as specific binding interactions could change the diffusion properties and the availability of free molecules. Their impact on reaction kinetics in the relevant context of living cells is still elusive, mainly due to the difficulty of capturing fast kinetics *in vivo*. This paper shows spatially resolved measurements of DNA hybridization kinetics in single living cells. HeLa cells were transfected with a FRET labeled dsDNA probe by lipofection. We characterized the hybridization reaction kinetics with a kinetic range of 10 μs to 1 s by a combination of laser-driven temperature oscillations and stroboscopic fluorescence imaging. The time constant of the hybridization depended on DNA concentration within individual cells and between cells. A quantitative analysis of the concentration dependence revealed several-fold accelerated kinetics as compared to free solution for a 16 bp probe and decelerated kinetics for a 12 bp probe. We did not find significant effects of crowding agents on the hybridization kinetics *in vitro*. Our results suggest that the reaction rates *in vivo* are specifically modulated by binding interactions for the two probes, possibly triggered by their different lengths. In general, the presented imaging modality of TOOL (Temperature Oscillation Optical Lock-in) microscopy allows to probe biomolecular interactions in different cell compartments in living cells for systems biology.

65-Plat
Dynamics of the HIV Reverse Transcription Initiation Complex
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Reverse transcription of the HIV genome begins from a ternary complex containing a tRNA primer, the highly-structured viral RNA template, and the reverse transcriptase (RT) enzyme. Previous work on RT revealed that RT binds its nucleic acid substrates in a variety of different modes and dynamically switches between these modes, for example, flipping between different binding orientations and sliding between alternate positions on the substrate. To assess RT's dynamics on more complicated substrates and to understand how these dynamics influence the initiation of reverse transcription, we employ a single molecule FRET assay to monitor the interactions between RT and the initiation tRNA/viral RNA complex. These measurements define the binding configuration of RT at each stage of the extension of the tRNA primer. We find that RT can bind the initiation complex in two orientations, corresponding to a productive orientation and an inactive orientation, and spontaneously flip between the two orientations. Both the composition of the primer's 3′ end and the secondary structure of the template mediate RT's binding orientation. As RT extends the tRNA primer, the amount of time it spends in the productive binding orientation first decreases, then increases as more nucleotides are added to the end of the tRNA primer. These results mirror ensemble primer extension assays showing that RT acts slowly and distributively during the addition of the first few nucleotides, then transitions to a fast, processive mode. These results demonstrate that RT's binding dynamics regulate the initiation of reverse transcription and provide a mechanistic explanation for the changes in RT activity during initiation.

66-Plat
DNA Unwinding By DnaB and the DnaB/TAU Complex
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The replicative helicase for E. coli is DnaB, a hexameric, ring-shaped motor protein that encircles and translocates along ssDNA, denaturing dsDNA in advance of its motion by sterically occluding the complementary strand to the outside of the ring. Using multiplexed single-molecule measurements with magnetic tweezers, we investigate the translocation and unwinding activities of DnaB. We find that DnaB's interaction with the ss/dsDNA junction is dependent on the geometry of the DNA substrate and applied force, suggesting that the hexamer interacts with the occluded strand during unwinding. We have also found that the structure of the bound nucleotides within DnaB's central channel is highly compact relative to the contour length of ssDNA, consistent with crystal structures of related hexameric helicases. Finally, in all our experiments, we find high variance in the rates of unwinding as well as frequent pausing, indicating that individual hexamers fluctuate among different conformations with different activities. To investigate DnaB's variable nature, we test the effect on helicase activity of interactions with the tau subunit of the Pol III holoenzyme, which is thought to regulate DnaB's unwinding rate.

67-Plat
Chemo-Mechanical Study of a Hexameric Helicase on the Single-Molecule Level
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Cell division involves DNA replication that requires the opening of double stranded DNA by cellular machines known as helicases. Although many of

those essential enzymes are of hexameric structure, not much is known about coordination and the mechano-chemical function of these multimeric enzymes. Here, we study with single-molecule FRET (Förster resonance energy transfer) a hexameric helicase of the DnaB family, called G40P. DnaB helicase is the essential replication helicase in prokaryotes and consists of 6 identical subunits that exhibit the widely shared RecA-fold in biological enzymes. In order to gain information about the chemo-mechanical cycle of G40P, we followed the time trajectory of individual enzymes while unwinding a DNA duplex. By the addition of the non-hydrolyzable ATP analogue ATPgS to the reaction at low ratio of ATPgS to ATP, we observed significant stalls during the unwinding process. Varying the concentration of ATPgS did not affect the lifetime of the stall, which indicates a strong coordination between the identical subunits. Based on this observation, we propose a highly coordinated subsequent ATP hydrolysis between the subunits, where binding ATPgS at a single site can stall the entire helicase. Furthermore, under suboptimal conditions like low ATP concentrations, we observed frequent repetitive slippage events of individual helicases, indicating a transient loss of tight binding to the DNA substrate.

68-Plat
Single Molecule Studies Revealing the Dynamics of RNA Helicase eIF4A
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Eukaryotic initiation factor eIF4A is a prototype protein of the DEAD box family of RNA helicases, and is part of the translation initiation complex eIF4F. eIF4A binds to the 5′ cap of mRNA and unwinds structures in the 5′-untranslated regions of mRNAs in ATP dependent manner. Our long-term goal in this project is to decipher the role of the initiation complex eIF4F in ribosomal recruitment, and develop methods to control this process. Although eIF4A has been studied extensively by classical bulk biochemical methods, a direct, unambiguous measurement of its helicase activity and its processivity has not been reported. Here, we use single molecule fluorescence assays to visualize its binding to RNA and melting secondary structures in RNA. Specifically, FRET efficiency dynamics is used to explore the binding location of eIF4A and its unwinding function. We demonstrate that eIF4A does not move on single stranded region, it preferentially binds at a close proximity to the single-strand (ss) / duplex junction on substrates with ssRNA overhangs. We seek to elucidate any elementary steps and kinetic mechanisms involved with eIF4A unwinding of RNA. Single-molecule FRET values decrease with a discrete pattern corresponding to the number of steps for unwinding. We observe the intermediate FRET states in various substrates and conclude that eIF4A unwinds 6 base pairs per step. The processivity of eIF4A increases in the presence of cofactors such as eIF4H. Furthermore, we selectively probe eIF4A activity with small-molecule inhibitor pateamine which stimulates eIF4A activity.

69-Plat
Regulation of PKR By Viral RNAS
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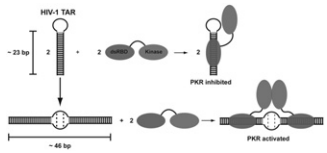
PKR is an interferon-induced kinase that plays a key role in the innate immunity response to viral infection. Upon binding dsRNA, PKR undergoes autophosphorylation reactions that activate the kinase. We have investigated the mechanism of PKR activation by two viral RNAs that regulate PKR. HIV-I TAR is a 23 bp RNA hairpin with three bulges that is known to dimerize. A single PKR binds with moderate affinity to TAR monomer whereas dimers bind two PKRs. TAR dimers activate PKR whereas monomers do not. The secondary structure defects in the TAR RNA stem function as antideterminants to PKR binding and activation. Our results support a model where dimerization of the TAR RNA hairpin facilitates sequential binding of two PKR monomers, leading to protein dimerization and subsequent activation. Adenovirus VAI is a 160 nt highly structured RNA that inhibits activation of PKR by dsRNA. The stoichiometry and affinity of PKR binding to VAI are regulated by Mg2+. In the presence of 5 mM Mg2+, PKR binds similarly to VAI and to a truncation mutant lacking the terminal stem, indicating that this region of VAI is dispensable for regulation of PKR activation.



Symposium 3: Multiscale Structural Analysis of Very Large Complexes

70-Symp
Mass Spectrometry and Its Contribution To Hybrid Structure Determination
Carol Robinson.
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Standard proteomics techniques are unable to describe the stoichiometry, subunit interactions and organization of assemblies since many are heterogeneous, present at low cellular abundance and frequently difficult to isolate. We have combined two existing methodologies to tackle these challenges: affinity purification and nanoflow ESI-MS. We use methods designed to maintain non-covalent complexes within the mass spectrometer to provide definitive evidence of interacting subunits based on the masses of complexes and subcomplexes generated by perturbation both in solution and gas phases. Structural models will be presented for oligomeric protein complexes with different degrees of structural information including the human U1snRNP and eIF3 complexes. These models will then be examined within the context of their function.
Recent developments in mass spectrometry have added a further dimension to our studies of protein complexes: that of their collision cross-section. Using ion mobility mass spectrometry we have been able to add spatial restraints to our models validating our models with measurements of collision cross-sections. Very recently we have had a considerable breakthrough which has enabled us to preserve intact membrane complexes in the gas phase. This enables us to establish lipid and nucleotide binding and to define the stoichiometry and post translational modifications within the intact transmembrane regions of a number of complexes. I will demonstrate some of the advantages of this approach by presenting recent insights into the structures of intact V-type ATP synthases.

71-Symp
Assembly of the 30s Ribosome From the RNA Folding Perspective
Sarah Woodson, Tadepalli Adilakshmi, Priya Ramaswamy, Sarah F.C. Soper, Deepti L. Bellur.
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Ribosome assembly requires folding of the rRNA and the hierarchical addition of 20 or more proteins to the complex. We visualized assembly of the bacterial 30S ribosomal subunit in real time using time-resolved hydroxyl radical footprinting. This method reveals the extent of RNA and protein interactions at each segment of the RNA backbone, providing a detailed view of the changes to the rRNA structure during assembly. Each domain of the 30S ribosome assembles concurrently in vitro, and many tertiary RNA interactions and RNA-protein interactions are established within the first 0.1 seconds. Individual proteins protect different segments of their binding site at different rates, suggesting that the initial protein-RNA complexes are remodeled during assembly. By perturbing the free energy of RNA-protein complexes from the body of the 30S subunit, we find that a single protein can stabilize an entire domain of the 16S rRNA. However, multiple proteins bound to the same domain narrow the ensemble of rRNA conformations. Specific structural switches stabilize the decoding active site and enable long-range structural communication within the 30S ribosomal subunit.

72-Symp
Nuclear Pore Complex Structure, Conservation and Plasticity
Ueli Aebi.
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No Abstract.

73-Symp
Integrating Diverse Data For Structure Determination of Macromolecular Assemblies
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Our broad goal is to contribute to a comprehensive structural characterization of large macromolecular assemblies. Detailed structural characterization of assemblies is generally impossible by any single existing experimental or computational method. We suggest that this barrier can be overcome by hybrid approaches that integrate data from diverse biochemical and biophysical experiments (eg, x-ray crystallography, NMR spectroscopy, electron microscopy, immuno-electron microscopy, footprinting, chemical cross-linking, FRET spectroscopy, small angle X-ray scattering, immunoprecipitation, and genetic interactions). Even a coarse characterization of the configuration of macromolecular components in a complex (ie, the molecular architecture) helps to elucidate the principles that underlie cellular processes, in addition to providing a necessary starting point for a higher resolution description.